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Chronic Pulsatile Shear Stress Impacts Synthesis of Proteoglycans by Endothelial Cells: Effect on Platelet Aggregation and Coagulation

Selim Elhadj,[1] Shaker A. Mousa,[2] and Kimberly Forsten-Williams[1]*

[1]Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061
[2]DuPont Pharmaceuticals Company, Experimental Station, Route 141 & Henry Clay Road, Wilmington, Delaware 19880

Abstract Endothelial-derived proteoglycans are important regulators of the coagulation-pathway in vivo and our primary objective of this study was to determine whether chronic shear stress affected the synthesis, release, and activity of proteoglycans from bovine aortic endothelial cells (BAEC). BAEC were cultured under shear and proteoglycans were purified from BAEC conditioned media and analyzed using both anionic exchange and size exclusion chromatography. The overall amount of proteoglycans produced per cell was significantly greater for the high shear-treated samples compared to the low shear-treated samples indicating that the shear magnitude did impact cell responsiveness. While overall size and composition of the proteoglycans and glycosaminoglycan (GAG) side chains were not altered by shear, the relative proportion of the high and low molecular weight species was inversely related to shear and differed significantly from that found under static tissue culture conditions. Moreover, a unique proteoglycan peak was identified from low shear stress (5 ± 2 dynes/cm^2) conditioned media when compared to high shear conditions (23 ± 8 dynes/cm^2) via anionic exchange chromatography, suggesting that subtle changes in the GAG structures may impact activity of these molecules. In order to characterize whether these changes impacted proteoglycan function, we studied the effects of shear specific proteoglycans on the inhibition of thrombin-induced human platelet aggregation as well as on platelet-fibrin clot dynamics. Proteoglycans from high shear-treated samples were less effective inhibitors of both platelet aggregation and blood coagulation inhibition than proteoglycans from low shear-treated samples and both were less effective than proteoglycans isolated from static tissue culture samples. However, due to changes in the overall proteoglycan synthesis and release rate, the high and low shear-treated sample had essentially identical effects on these activities, suggesting that the cells were able to compensate for stress-induced proteoglycan changes. Our data suggests that shear stress, by altering proteoglycan synthesis and fine structure, may play a role in maintaining vascular hemodynamics and hemostasis. J. Cell. Biochem. 86: 239–250, 2002.

Key words: glycosaminoglycan; proteoglycans; shear; platelets; vascular

Endothelial cells constitute a natural active boundary between blood and the underlying vascular tissue. Under normal conditions, transluminal pressure gradients and stretch forces act on the entire vascular wall, including underlying fibroblast and smooth muscle cells (SMC), while shear stress acts primarily on the endothelium [Liu, 1999]. Hemodynamic forces represent an essential modulator of vascular function via, in part, release of active molecules by endothelial cells targeted to control vasomotor tone, wall remodeling, and cell to cell interactions [Davies, 1995]. Ultimately, the goal is to maintain hemostasis and to sustain normal baseline functional vascular responses to the pervasive hemodynamic environment. Disturbed flow conditions are prevalent in vascular bifurcations and following surgical

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*Correspondence to: Kimberly Forsten-Williams, Department of Chemical Engineering, 133 Randolph Hall, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061. E-mail: kforsten@vt.edu

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interventions [DeBakey et al., 1985; Asakura and Karino, 1990]. These disturbances often include significant spatial and temporal gradients [Davies et al., 1995], regions of recirculation [Ravensbergen et al., 1998], low average shear stresses [Gibson et al., 1993; Chiu et al., 1998; Jiang et al., 1999], and transient back flow [Buchanan et al., 1999]. While systemic factors contribute to vascular disease, a strong positive correlation exists between these local hemodynamic conditions and the focal nature of atherosclerotic plaque formation [Eskin and McIntire, 1988]. Platelet aggregation and activation following plaque disruption is an important step in the pathology of acute cardiovascular disease [Libby, 2000; Fitzgerald, 2001].

Numerous studies document the relation between shear stress and the release of vasoactive agents [Davies, 1995]. For example, prostaglandin I_2, a potent SMC growth inhibitor, platelet aggregation inhibitor, and vasodilator, is transiently upregulated by increasing shear stress [Okahara et al., 1998]. In contrast, increasing shear stress downregulates endothelin-1 [Redmond et al., 1997], which has the opposite effects of promoting SMC growth [Davies, 1995] and increasing vessel vasoconstriction [Marsden et al., 1989]. Further, nitric oxide is part of the early response to increased shear stress as a SMC growth inhibitor and by augmenting vessel luminal diameter [Cornwell et al., 1994]. In a non-compromised vasculature, these vasoactive agents tend to maintain a "setpoint" shear stress, acutely via the release of vasomotor agents and chronically by modulating vessel wall remodeling [Davies, 1995]. In this study, we have focused on chronic shear stress treatment and the effects on bovine aortic endothelial cells (BAEC) synthesis of proteoglycans.

Proteoglycans are ubiquitous glycoproteins that are composed of a protein core with sulfated glycosaminoglycans (GAGs) side chains as their main constituents [Jackson et al., 1991; Kjellen and Lindahl, 1991]. These highly negatively-charged GAGs are important for the interactions of proteoglycans with growth factors and enzymes involved in the coagulation pathway [Bourin and Lindahl, 1993]. Proteoglycans have been implicated in the process of intimal hyperplasia and thrombosis [McCarty, 1997], both major events in atherosclerosis development. Further, both cell surface associated and releasable proteoglycans as well as the GAG heparin can be effective coagulation inhibitors in vivo [Nader et al., 2001]. For example, within the intrinsic coagulation pathway, proteoglycans can inhibit thrombus formation by activating various serpins or protease inhibitors, including antithrombin III [Bourin and Lindahl, 1993], heparin co-factor II [Jackson et al., 1991], and leuserpin [Jackson et al., 1991]. Platelet-collagen interactions have been shown to be inhibited by soluble proteoglycans [Lassila et al., 1997] and proteoglycans have been shown to specifically inhibit thrombin-induced platelet aggregation [Luzzatto et al., 1989; Nakashima et al., 1992]. Endothelial-derived proteoglycans have a role in normal vascular homeostasis.

Two previous studies have examined proteoglycan metabolism under conditions of non-pulsatile sustained laminar flow. Grimm et al. [1988] found that proteoglycans were differentially polarized amongst the medium, plasma membrane, and matrix when BAEC were exposed to flow for 2 h, noting that the chromatograph profiles of the proteoglycans resembled those found in vivo as shear rate was increased. Arisaka et al. [1995] studied proteoglycan metabolism from porcine endothelial cells post shear stress treatment and found that, in contradiction with Grimm and co-workers, GAG synthesis increased with shear stress. Both experiments focused on the effect of shear treatment for 24 h or less and, hence, were focused on acute or short-term responses. In addition, both used steady non-pulsatile flow. In contrast, our experimental model is geared toward the study of the release of the proteoglycans from BAEC under longer-term physiological flow rates using a pulsatile laminar flow.

In our studies, we used the Cellmax® capillary system that is based on endothelial cell culture on the walls of hollow fibers having the general dimensions of blood vessels [Redmond et al., 1995]. With this model, we cultured BAEC under pulsatile flow conditions, resulting in shear stresses of either 5 ± 2 or 23 ± 8 dynes/cm^2 (mean $\pm$ amplitude) in order to ascertain the effects of shear stress on endothelial cell metabolism of proteoglycans. We compared our flow system proteoglycans with samples isolated from endothelial cells cultured in the absence of flow in tissue culture conditions typical of most in vitro endothelial cell studies. Our primary goal was to evaluate how these proteoglycans impact platelet aggregation as an indicator of the possible effect on hemostasis. Our data show that shear stress can alter both the

quantity and fine structure of proteoglycans released from BAEC, which ultimately can regulate platelet activity and coagulation in whole blood.

MATERIALS AND METHODS

Materials

BAEC, cryopreserved at passage 8, were obtained from the Coriell Institute (Camden, NJ). All chromatography supplies and general chemicals as well as dimethylmethylene blue dye (DMB), blue dextran (2,000,000 MW), human thrombin, chondroitinase ABC (C.ABC), and unfractionated heparin (ovine intestinal mucosa) were purchased from Sigma-Aldrich (St. Louis, MO). Heparinase III (Hep III) was a generous gift from IBEX Pharmaceuticals (Montreal, Canada). Beef lung unfractionated heparin used for the aggregation studies was purchased from Pharmacia & Upjohn (Peapack, NJ). Recombinant human tissue factor was purchased from Dade Behring (Deerfield, IL). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Media supplements with the exception of acidic fibroblast growth factor (aFGF) and all tissue culture supplies were purchased from Fisher Scientific (Suwanee, GA). aFGF was obtained from Gibco-BRL Life Technologies (Carlsbad, CA). Aqueous sodium sulfate ($Na_2{}^{35}SO_4$) was purchased from NEN Life Science (Boston, MA). Dialysis cassettes (3,500 MWCO) were from Pierce (Rockford, IL). The Cellmax® High Flow Quad Artificial Capillary Cell Culture System was from Cellco® Spectrum Laboratories (Rancho Dominguez, CA) and the hollow-fiber cartridges used were made of porous polypropylene (0.5-μm pore size) and were coated by the manufacturer with pronectin-F™ to facilitate cell adhesion.

Conditioned Media Collection

BAEC, passage 10, were seeded on the luminal surface of pronectin-F coated polypropylene capillaries contained in a sealed cartridge at 2.9×10^4 cells/cm^2 as described previously [Redmond et al., 1995]. A total of 50 capillaries (9.6 cm long, 330 μm ID, 150 μm wall, 0.5 μm pore size) are contained within each cartridge and the system can handle four independent cartridges simultaneously. Culture media (Ham's F12, supplemented with 10% FBS, 1% L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and aFGF (3.5×10^{-3} μg/ml) was directed within the extra-capillary space to allow for cell attachment for 24 h before initiating exposure of the cell culture to flow (4 ml/min). Seeding efficiency in the cartridges was ~85%. The cells were cultured for 7 days under nominal shear stress (< 0.5 dynes/cm^2) before gradually ramping up the flow rate to obtain the desired shear rates. The culture was maintained for a total of 14 days including the treatment shear stresses (final 72 h). Prior to conditioning, a 1-h wash with serum-free Ham's F-12 media was done. Conditioning with $Na_2{}^{35}SO_4$ (30 μCi/ml) supplemented serum-free Ham's F-12 at the desired flow rate followed. The conditioned media was collected 24 h later and centrifuged (Jouan CR412, Wincester, VA) at 1,865*g* for 10 min at 4°C to remove any cell debris. The treatment shear stresses were 5 ± 2 and 23 ± 8 dynes/cm^2 (mean shear stress ± amplitude of shear resulting from pulsatile flow). The pulsatile flows had similar frequencies for low and high shear treatments of ~0.3 Hz and were essentially sinusoidal (data not shown). The intraluminal average pressure gradients across the cartridges for low and high shear cultures were 9.6 and 26 mm of Hg, respectively. The non-flow cultures involved seeding BAEC on polypropylene membranes placed in 12-well tissue culture plates at an identical seeding density as used in shear stress cultures. Cells were grown for 7 days including the 1-h wash and 24-h conditioning with $Na_2{}^{35}SO_4$ (30 μCi/mL). Following collection, all conditioned media was centrifuged to remove any cell debris, and processed for purification as described below. Purified samples were frozen at −80°C and thawed as needed.

Isolation of Proteoglycans and GAGs

Isolation of proteoglycans from conditioned media was performed as described previously [Forsten et al., 1997]. Briefly, urea (1 mol/L) was added to the conditioned media prior to loading on a Q-sepharose anionic exchange column (1.5×3.5 cm) at 1 ml/min. A step salt gradient (Tris buffer, (50 mmol/L Tris, pH 8.0)), 0.3 mol/L NaCl) was followed by the elution of the target proteoglycans using Tris buffer, 1.5 mol/L NaCl. Flow rates were maintained at 1.0 mL/min for all elutions.

Dialysis against TBS (Tris buffer, 0.15 mol/L NaCl) using dialysis cassettes (MWCO 3,500) was performed to remove urea, any unincorpo-

rated sulfate, and to reduce salt concentration. Proteoglycan fractions from shear treated BAEC were concentrated by lyophilization. Concentrations were determined in triplicates using the DMB spectrophotometric assay [Farndale et al., 1986] with beef lung or ovine intestinal mucosa unfractionated heparins as standards. Media, not exposed to cell cultures, were subjected to the same purification and concentration protocol to generate control samples. GAGs from purified proteoglycans fraction were obtained using an alkaline β-elimination reaction as described previously [Bassols and Massague, 1988]. Briefly, purified proteoglycans were incubated for 24 h at 37°C in 1 mol/L $NaBH_4$, 0.05 mol/L NaOH at pH 12.5. Glacial acetic acid was used to quench the solution to pH 7.0.

Enzymatic Digestion

Proteoglycan fractions were analyzed using differential enzyme digestion with Hep III and C. ABC as described previously [Forsten et al., 1997]. Hep III was used at working concentration of 0.25 U/ml and C.ABC at 1 U/ml. Tris-HCl buffer (50 mmol/L Tris, pH 7.52, 0.15 mol/L NaCl) was prepared for all digestions. Samples were incubated with Hep III alone, C.ABC alone, or both enzymes for 4 h at 37°C and then assayed using spectrophotometry and DMB. Control samples were incubated without enzymes.

Chromatography

[$^{35}SO_4$]-incorporated proteoglycans and GAGs were passed over Sepharose CL-2B (1.0 × 40 cm) and Sepharose CL-6B (1.0 × 40 cm) size exclusion columns equilibrated in TBS at an elution flow rate of 1 ml/min. The radioactivity per fraction (1 ml) was determined using a Packard Liquid Scintillation Analyzer 2100 TR (Meriden, CT). The void and total volumes of the columns were determined using blue dextran and $Na_2{}^{35}SO_4$, respectively. Average MW of proteoglycans and GAGs were obtained from derived correlations based on the K_{av} values [Wasteson, 1971; Ohno et al., 1986].

We also used a DEAE ion exchange column (1.0 × 3.5 cm) as the stationary phase to characterize elution profiles of isolated proteoglycans and GAGs. Samples were loaded and eluted using a linear gradient (0.15–1.5 mol/L NaCl) in 95 min at 1 ml/min with fractions collected every minute. The eluent stream was monitored for its conductance with a Bio-Rad conductance meter EG1 Gradient Monitor (Hercules, CA). Samples were quantified using a liquid scintillation analyzer.

Platelet Aggregation Studies

Blood samples were obtained fresh from a healthy male human donor who had not taken aspirin for at least 2 weeks prior to blood collection. Venous blood was collected in sodium citrate (3.2%) vacutainers tubes. The platelet-rich-plasma (PRP) fraction was extracted following centrifugation at 150*g* for 10 min with a Sorvall RT6000 Tabletop centrifuge with H-1000 rotor followed by isolation of the platelet-poor-plasma (PPP) fraction by centrifugation (1,500*g* for 10 min). The PPP was used to dilute and scale the PRP to $\sim 4.0 \times 10^8$ platelet/ml for all samples used in the aggregation study. Blood and PPP were kept at room temperature during the study.

A Bio/Data Corporation aggregometer PAP-4 (Horsham, PA) was used to determine steady state values of percent platelet aggregation. PRP (200 μl) was mixed with isolated proteoglycan material (20 μl) and equilibrated at 37°C for 8 min prior to thrombin (0.5 IU/ml, 20 μl) addition. Readings were taken continuously for at least 4 min per sample. PPP was used as a control for 100% transmittance. Dose-response curves for thrombin-induced aggregation were generated (data not shown) to determine maximal stimulation.

Thromboelastography Studies

A computerized Thromboelastograph® (CTEG Model 3000; Haemoscope Corp., Skokie, IL) was used to determine the peak rigidity of blood samples treated with shear specific-proteoglycan fractions as described previously [Mousa, 2000]. Briefly, torque measurements on a suspended stationary piston residing inside of an oscillating cup containing whole blood and treatment samples were recorded at 37°C with tissue factor (4 μg/mL) as a coagulation agonist and $CaCl_2$ (3 mmol/L) as a coagulation cofactor. As the fibrin/platelet fibrils form, peak-to-peak torque increases indicating an increase in overall rigidity. Blood sample elasticity measurements resulting from proteoglycan treatment was referenced to that of negative controls (media treated identically to the purified proteoglycans) to determine the % inhibition of the samples.

Statistics

Means were compared for significance using the Method of Contrasts with SAS statistical software (SAS Institute).

RESULTS

Experimental Biomechanical Model

The flow system used for our shear stress studies has been previously described by Redmond et al. [1995]. The system is composed of a control unit that regulates four independent positive displacement pumps that are connected to four independent cell cartridges. The system allows for the culture of endothelial cells under pulsatile flow for prolonged period of time and, thus, more closely replicates the chronic shear stress conditions encountered by endothelial cells in vivo than does typical tissue culture conditions. Chronic shear stress treatment of BAEC was achieved by seeding cells inside porous polypropylene capillaries contained within a sealed plastic casing. The flow rate was steadily increased over a period of 7 days following the initial 7 days of growth at a nominal shear stress (< 0.5 dynes/cm^2). Cells were subjected to the desired physiological shear stress levels during the last 72 h of shear treatment: low shear (5 ± 2 dynes/cm^2) or high shear (23 ± 8 dynes/cm^2) (mean $\pm$ amplitude). The shear stresses used are typical of venous type shear stresses (low shear) and arterial type shear stresses (high shear) and were calculated using measured pressure drop, flow rate, and fluid viscosity data [Womersley, 1955]. Flow inside the capillaries was pulsatile and nearly sinusoidal (data not shown). Both the pressure waveform frequencies ($\sim$0.3 Hz) and the ratio of amplitude to average shear stress were similar in our low and high shear experiments (0.4 and 0.35, respectively). BAEC cultured on similar polypropylene membranes maintained in static culture plates were used as a comparison between our model system and more typical tissue culture conditions used in endothelial studies.

Effects of Shear Stress on BAEC-Released Proteoglycans and GAGs Levels

The effect of short-term (≤ 24 h) shear stress on endothelial cell synthesis of proteoglycans has been studied [Grimm et al., 1988; Arisaka et al., 1995] with conflicting results, however, our focus was on how chronic shear stress (> 48 h) impacts regulation of these molecules. Due to the difficulties in extracting cellular and extracellular matrix proteoglycans from our hollow fiber system, we focused on soluble/released material which could be purified from conditioned media and was comparable to materials collected by previous investigators [Grimm et al., 1988; Arisaka et al., 1995; Forsten et al., 1997]. Following purification from conditioned media, overall levels of sulfated proteoglycans/GAGs were determined using the DMB spectrophotometric assay [Farndale et al., 1986]. Shear stress led to a significant increase in proteoglycan levels ($P < 0.005$) when compared to typical tissue culture samples (Table I). Further, the higher average shear stress samples had significantly higher levels ($P < 0.01$) of proteoglycan/GAG than did the low shear sample. Control media not exposed to BAEC had no detectable proteoglycans. Despite changes in overall levels, shear stress did not appear to affect overall GAG composition. Heparan sulfate GAG chains constituted $\sim$80% of the total GAG content with chondroitin/dermatan sulfate GAG chains making up the balance for all

TABLE I. Bulk Characterization of Proteoglycans/GAGs Isolated From Conditioned Media

Treatment	Proteoglycan ($\mu g \times 10^5$/ng DNA)	HS (%)	CS/DS (%)
No shear tissue culture	1.37 ± 0.37	84.1 ± 5.5	18.5 ± 3.6
Low shear (5 ± 2 dynes/cm^2)	3.95 ± 0.41[a]	82.0 ± 6.3	21.5 ± 3.2
High shear (23 ± 8 dynes/cm^2)	5.59 ± 0.36[a,b]	80.4 ± 4.6	19.3 ± 5.0

[a]$P < 0.005$ vs. no shear.
[b]$P < 0.01$ vs. low shear.
The amount of proteoglycans followed a similar trend when normalized to cell number (data not shown). Proteoglycans were purified and treated as described under Materials and Methods and amounts determined using a DMB spectrophotometric assay. Proteoglycan amounts were normalized to total DNA. GAG composition was determined, based on difference assay of DMB readings: heparan sulfate (control (no enzyme)-Hep III), chondroitin/dermatan sulfate (control-C.ABC). Error bars represent SD for the results of three independent experiments (n = 3).

three samples. The GAG ratios are essentially identical to those found previously for BAEC cultured on tissue-culture plastic under static conditions [Forsten et al., 1997].

Effect of Shear Stress on BAEC-Released Proteoglycan Size

Changes in overall size of the proteoglycan and GAG sidechains have been shown to impact activity [Bourin and Lindahl, 1993; Forsten et al., 1997] and, thus, we investigated whether shear stress affected either of these important characteristics. Following purification from conditioned media, proteoglycan fractions were separated over a CL-2B Sepharose size exclusion chromatography column. In all cases, two broad peaks were evident with a Kav values of 0.14 and 0.48, corresponding to average molecular weights of 2.2×10^6 and 6.1×10^5, respectively [Wasteson, 1971; Ohno et al., 1986] (Fig. 1). The elution peaks occurred at the same Kav value for all samples, however, the proportion of high to low molecular weight species decreased with shear. Specifically, the percentage of high-molecular weight fraction decreased from $30 \pm 3\%$ for non-shear samples to $23 \pm 2\%$ for low shear and $19 \pm 2\%$ for high shear samples (mean $\pm$ SE based on n = three independent runs from three independent isolations). Although in each of three independent experiments, the high shear treatment led to a lower percentage of high molecular weight material than the low shear sample, variations between experiments did not allow for a significant difference to be shown based on an average percentage. The difference between shear and static culture samples was significantly different, however, even with the slight variability ($P < 0.05$).

To determine whether these differences in distribution were reflected in changes in the overall GAG size profile, proteoglycan fractions were treated with alkaline borohydride to release the GAG chains and the material was separated over a Sepharose CL-6B column. Borohydride treatment was effective in releasing the GAG side chains as undigested fractions showed only a single peak near the void fraction (data not shown). A primary peak at a Kav of 0.43 (MW 3.0×10^4) and a secondary smaller fraction at a Kav of 0.83 (MW 3.5×10^3) were found for the borohydride-treated samples (Fig. 2). Shear stress does not appear to affect either the distribution or the elution of the GAG

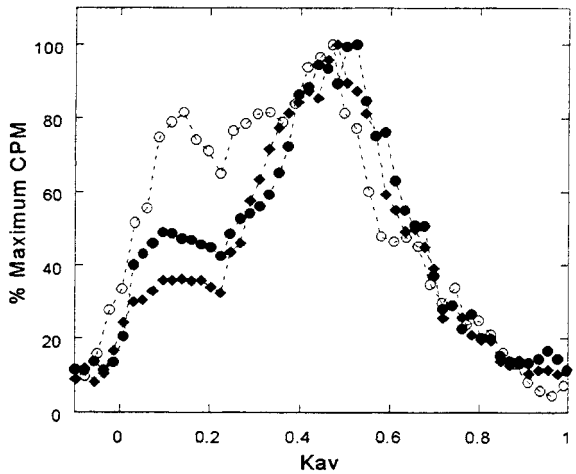

Fig. 1. Elution profiles of isolated proteoglycans from the conditioned media of shear and non-shear treated BAEC. Metabolically labeled [^{35}S]proteoglycans were purified as described under Materials and Methods from the conditioned media of shear treated and static cultures of BAEC. Samples were separated on a Sepharose CL-2B column and radioactivity for each fraction was determined. The results were normalized to the maximum counts obtained from the fractions eluted. No shear (○), low shear (●), and high shear (◆) treated samples represent shear stress treatments of 0, 5 ± 2, and 23 ± 8 dynes/cm^2, respectively. Elution profiles are representative of profiles from three independent cell culture experiments.

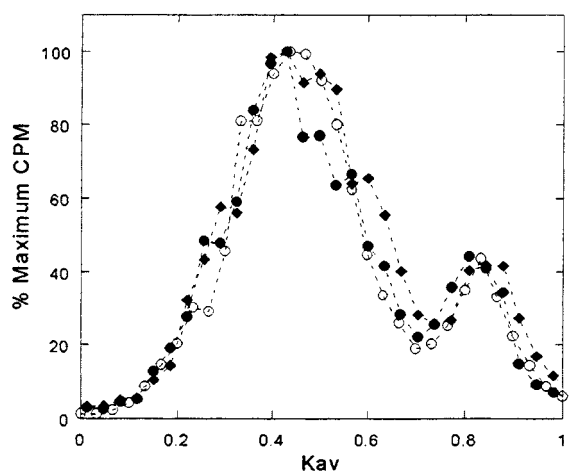

Fig. 2. Sepharose CL-6B elution profiles of GAGs from the conditioned media of shear and non-shear treated BAEC. Metabolically labeled [^{35}S]proteoglycans were purified as described under Materials and Methods from the conditioned media of shear treated and static cultures of BAEC. Following alkaline borohydride treatment GAGs were separated on a Sepharose CL-6B column and each fraction radioactivity was determined. The results were normalized to the maximum radioactivity counts obtained from the fractions eluted. No shear (○), low shear (●), and high shear (◆) treated samples represent shear stress treatments of 0, 5 ± 2, and 23 ± 8 dynes/cm^2, respectively. Elution profiles are representative of profiles from three independent cell culture experiments.

as the profiles were essentially identical for high and low shear samples as well as the no shear tissue culture material.

Effect of Shear Stress on BAEC-Released Proteoglycan Ion Exchange Profile

The extent of proteoglycan sulfation can impact function [Prydz and Dalen, 2000] and may reflect changes in the overall negative charge associated with the GAG sidechains [Bourin and Lindahl, 1993]. Hence, we used ion exchange chromatography to separate proteoglycan and GAG fractions based on their ionic binding affinity as a means of characterizing shear stress induced effects on the fine structure of the proteoglycans.

The elution pattern of the proteoglycan sample from the low shear-treated cells showed three peaks at 0.30, 0.46, and 0.62 mol/L NaCl (Fig. 3A). The proteoglycan sample from the high shear-treated cells had a similar elution pattern with the exception of the low salt fraction (0.30 mol/L). This fraction was essentially absent from both the high shear sample as well as the no shear static culture sample with less than 3% of the total material found in these representative fractions (Table II). This peak did not correspond to unincorporated sulfate as this eluted at 0.15 mol/L NaCl (data not shown) nor do we believe this is a free GAG as borohydride treatment of the samples did not show a fraction that eluted at this salt concentration (Fig. 3B). For all samples, the majority of the material eluted in the high salt peak.

Three main peaks were found for the borohydride-treated samples corresponding to salt concentration of 0.15, 0.42, and 0.59 mol/L (Fig. 3B). While low shear and high shear GAGs had a similar peak distribution, the no shear static culture samples had a distinct pattern that differed from the shear samples. Specifically, there was a higher ratio of the earlier eluting peaks than was found for the shear-treated samples (Table II), indicating a difference in the fine structure of the released GAG chains when BAEC are subjected to shear as opposed to when the cells are grown in static cultures. It should be noted, however, that the static cultures were maintained for only 7 instead of 14 days and that the membranes used were of the same base material but lacked the pronectin-F coating applied by the manufacturer of the hollow fiber cartridges. We cannot rule out the possibility that these factors impacted the resulting differences seen.

Differential Inhibition of Platelet Aggregation by Proteoglycans

Shear stress treatment of BAEC did impact the released proteoglycans as characterized by ion-exchange chromatography, suggesting that proteoglycan-mediated activity, such as inhibition of platelet activation, might be shear regulated. Heparin, a GAG similar in structure to heparan sulfate, has found clinic use due to its ability to reduce thrombosis in vivo and inhibit blood coagulation [Mousa, 2000; Zaman et al., 2000]. We examined the effects of purified shear specific proteoglycans on thrombin-induced platelet aggregation using light transmittance aggregometry and found that platelet aggregation was inhibited by purified proteoglycans

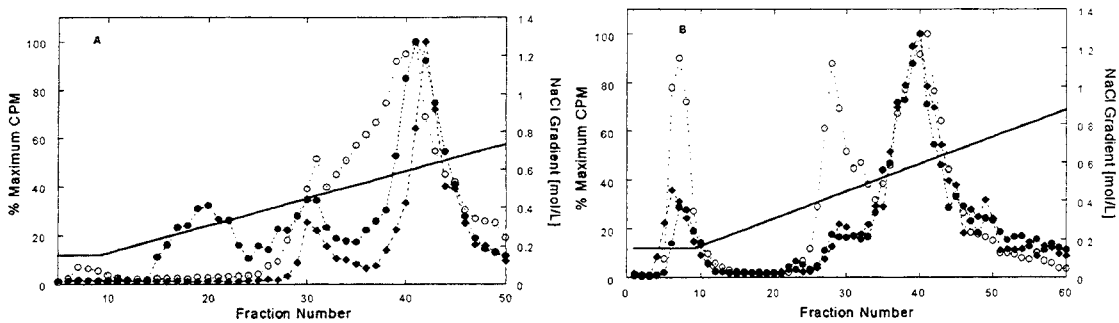

Fig. 3. DEAE ion-exchange column elution profiles of proteoglycans (**A**) and shear specific GAGs (**B**) from the conditioned media of shear and non-shear treated BAEC. Metabolically labeled [^{35}S]proteoglycans were purified as described under Materials and Methods from the conditioned media of shear treated and static cultures of BAEC. GAGs samples from β-eliminated proteoglycans and intact proteoglycans were eluted using a NaCl gradient (—) buffer and each fraction radioactivity was determined with a scintillation analyzer. The results were normalized to the maximum radioactivity counts obtained from the fractions eluted. No shear (○), low shear (●), and high shear (◆) treated samples represent shear stress treatments of 0, 5 ± 2, and 23 ± 8 dynes/cm^2, respectively. Elution profiles are representative of profiles from three independent cell culture experiments.

TABLE II. Peak Distributions From DEAE Ion Exchange Chromatography Separation of Proteoglycans and Borohydride Treated Proteoglycans

Peak location (mol/L, NaCl)	Static (%)	Low shear (%)	High shear (%)
Proteoglycans			
0.30	2 ± 1	18 ± 3	2 ± 1
0.46	13 ± 3	19 ± 3	18 ± 4
0.62	83 ± 5	62 ± 7	79 ± 5
Borohydride treated proteoglycans			
0.15	18 ± 3	10 ± 4	12 ± 2
0.42	28 ± 5	10 ± 3	9 ± 1
0.59	52 ± 8	79 ± 6	77 ± 4

Peak locations correspond with those shown in Figure 3. Metabolically-labeled [^{35}S]proteoglycans were purified as described under Materials and Methods from the conditioned media of shear treated and static cultures of BAEC. Samples were separated on a DEAE ion-exchange column and radioactivity for each fraction was determined. Error bars represent SD for the results of independent measurements from samples obtained from three independent cell culture experiments ($n = 3$).

from all samples (Fig. 4). The effect was specific to the cell-secreted material, as processed plain media had no inhibitory effect (data not shown). Proteoglycan samples from high-shear treated cells were less potent in inhibiting thrombin induced platelet aggregation (IC_{50} 0.24 μg/dose) when compared to proteoglycans from low shear-treated cells (IC_{50} 0.20 μg/dose), while proteoglycans from static culture samples were the most potent on a standard scale (IC_{50} 0.15 μg/dose). Although the low salt fraction (0.20 mol/L) found only in the low shear-treated samples using DEAE may be important with regard to function, we were unable to isolate a sufficient quantity for further characterization and activity measurements were done only using the purified unfractionated-proteoglycan samples.

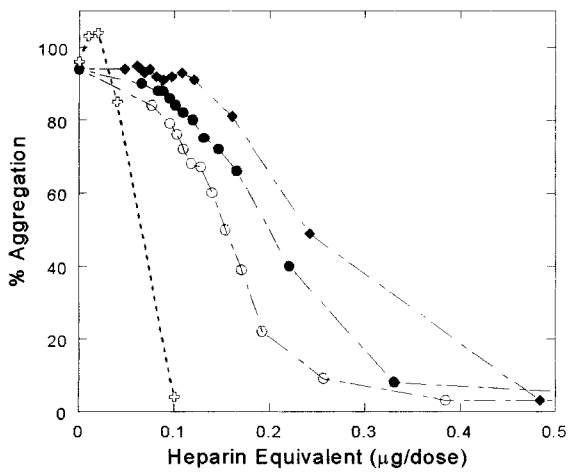

Fig. 4. Dose-response curves of thrombin induced platelet aggregation versus shear specific proteoglycans from the conditioned media of BAEC. Proteoglycans were purified and tested as described under Materials and Methods. The following IC_{50} were obtained: no shear (○) 0.15 μg/dose, low shear (●) 0.20 μg/dose, high shear (◆) 0.24 μg/dose, and heparin (+) (beef lung) 0.065 μg/dose. Data is representative of two independent experiments.

Differential Inhibition of Clot Formation by Proteoglycans

As an extension of our aggregometry data, we used a Thromboelastograph® to analyze proteoglycan inhibition of blood clot formation. Tissue factor (4 μg/ml) induced blood clot formation dose response curves were generated and a similar trend to that seen with the inhibition of platelet aggregation was found (Fig. 5). Specifically, proteoglycan samples from static culture samples were the most potent inhibitors of blood clot formation (IC_{50} 0.078 μg/dose) followed by the proteoglycans from low shear samples (IC_{50} 0.19 μg/dose). Proteoglycans from high shear samples (IC_{50} 0.30 μg/dose) were the least potent. Medium, which had not been exposed to BAEC but had been processed in parallel with the conditioned media, had no significant effect on clot formation (data not shown).

DISCUSSION

Mechanical forces, such as fluid shear, can impact cell activity and are thought to play a role in vascular disorders, such as atherosclerosis. For example, atherosclerotic plaques are more prominent in locations of disturbed blood flow suggesting that fluid flow may play a role in the pathology [Gibson et al., 1993; Irace et al., 1999; Jiang et al., 1999; Malek et al., 1999; Pedersen et al., 1999; Bakker and Gans, 2000]. There are clearly many biological factors that might find altered expression by endothelial cells in response to flow that would be important for vascular homeostasis. For example, both

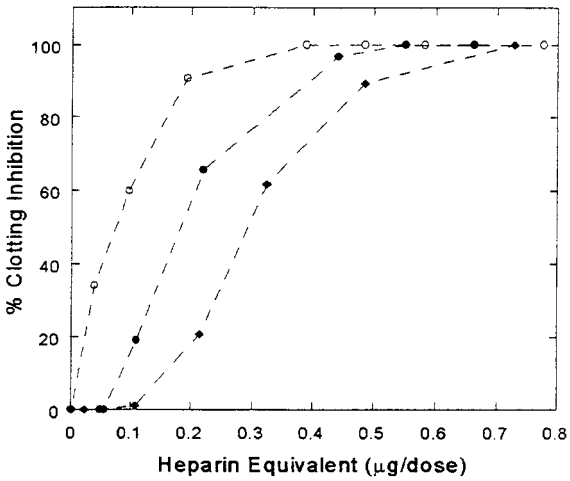

Fig. 5. Dose-response curves for tissue factor induced blood clot formation versus shear specific proteoglycans from the conditioned media of BAEC. Proteoglycans were purified and tested as described under Materials and Methods. Unexposed media controls did not inhibit significantly aggregation under the same conditions. The following IC_{50} were obtained: no shear (○) 0.080 μg/dose, low shear (●) 0.18 μg/dose, and high shear (◆) 0.30 μg/dose. Data is representative of two independent experiments.

message and protein levels of the cytokine interleukin-8 are decreased by steady flow laminar shear stress [Kato et al., 2001]. But interleukin-8 is simply one of many molecules for which availability can be altered by shear. Recent work by Chen et al. [2001] demonstated, using microarray technology, that many specific genes related to proliferation, remodeling, signal transduction, and inflammatory response were all altered by laminar flow. Specific means by which this regulation occurs is unclear, although studies have implicated activation of integrins by shear [Tzima et al., 2001] as well as production of regulatory compounds, such as nitric oxide [Go et al., 2001] or reactive oxygen species [Hsieh et al., 1998] and activation of ion channels [Nilius and Droogmans, 2001]. Since the endothelium is known to play an important role in vascular homeostasis and in the anticoagulation properties of the vasculature, our initial study presented here focused on the simple question of whether changes in the magnitude of the shear stress altered proteoglycan metabolism by endothelial cells.

We found several quantifiable differences between proteoglycans isolated from conditioned media from low shear (5 ± 2 dynes/cm^2) versus high shear (23 ± 8 dynes/cm^2) endothelial cells. The primary difference appears to be the level of proteoglycan production. We found significantly higher levels of proteoglycan from the high-shear samples than the low shear and both were higher than the static cell culture model (Table I). This change in overall proteoglycan production rate was not completely unexpected. Arisaka et al. [1995] saw an increase in GAG synthesis from porcine endothelial cells following 24 h of exposure to shear stress at both 15 and 40 dynes/cm^2. This was, however, in contract to work by Grimm et al. [1988] who found an inhibition of GAG synthesis, when BAEC were subjected to 1 dyne/cm^2 shear stress for 2 h. The work by Arisaka and co-workers suggested that a minimum exposure time to shear of 24 h was needed to see enhancement and our work looking at long-term exposure to shear certainly supports this, since we do see enhancement with the bovine cells. While the Cellmax experimental model does have the advantage of looking at cells cultured under shear for long periods of time and has the cylindrical geometry more representative of blood vessel architecture than flat parallel plate models, access to intact cell layer and extracellular matrix material is difficult and we, hence, restricted our study to released proteoglycans found in the medium. It is important to note that despite overall similarities between the tissue culture and the shear-treated samples, there were subtle differences in the ionic-exchange chromatography profiles (Fig. 3) between the shear- and non-shear-treated samples which likely impact the difference in activity (Figs. 4 and 5). Certainly there is increasing interest in defining fine-structural differences in proteoglycans due to the relevance to pathological conditions [Derwin et al., 2001; Plaas et al., 2001] and our work suggests that shear magnitude can affect those types of features.

The reports by Arisaka et al. [1995] and Grimm et al. [1988] are the only previous reports we have been able to identify addressing shear stress and proteoglycan/GAG synthesis. An additional significant difference between our study and those previous studies is the type of flow system used. In both earlier works, a steady laminar flow system was used. We, in contrast, have used a pulsatile flow system. Previous reports have indicated that pulsatile flow can lead to significant changes in endothelial cell morphology compared to either static flow or steady flow system [Helmlinger et al., 1991] and that cell activation [Levesque et al., 1990; Wittstein et al., 2000] and gene expression

[Noris et al., 1995; Lum et al., 2000; Hsiai et al., 2001] can differ. For example, Wittstein et al. [2000] report opposite effects on bovine endothelial cytosolic acidification when cells are exposed to steady versus pulsatile flow, indicating activation of different signaling pathways. Neither our work nor the previous work, examining shear and proteoglycan production, included a cyclic stretch component although certainly this can impact endothelial cell gene expression [Okada et al., 1998; Stula et al., 2000; Wang et al., 2001] and could be considered in future studies.

Given that the pulsatile nature of our flow at both high and low shear was essentially the same (~0.3 Hz) and that the system does not exhibit cyclic stretch, our study directly addresses the effect of differences in shear magnitude on characteristics of the proteoglycan production by endothelial cells. While the overall size of the proteoglycan fractions, based on separation over a Sepharose CL-2B size exclusion chromatography column, was unchanged, the ratio of the high-molecular-weight fraction to the low-molecular-weight fraction, in samples collected from three independent experiments, was higher for the low shear-treated compared to the high shear-treated material (Fig. 1). That this higher molecular weight fraction maybe more potent is suggested by our studies focused on platelet aggregation (Fig. 4) and clot formation (Fig. 5). In both assays, the low shear sample was a better inhibitor than the high shear material on an equal mass basis. Previously, we [Forsten et al., 1997] have shown that the higher molecular weight proteoglycan fraction from endothelial cells is more potent in inhibiting fibroblast growth factor-2 (FGF-2) mediated vascular smooth muscle cell binding and growth suggesting that shear-regulation of endothelial cell proteoglycans may also impact other proteoglycan-mediated vascular activities, such as the regulation of heparin-binding growth factors.

We have found both structural and overall quantity/synthesis difference in proteoglycans isolated from cells cultured under low and high shear stress. These structural changes manifest in differences in activity with regard to inhibition of platelet aggregation and clot formation. We also found, however, a difference in synthesis rate of proteoglycans from cells cultured under the high and low shear stress. If we, therefore, looked at the level of activity on a per cell basis, the differences in effect on aggregation and clot formation disappear and the curves are essentially superimposable (data not shown). This suggests that either the effect of shear stress on the proteoglycan fine structure is being compensated for by a change in proteoglycan synthesis per cell or that the change in synthesis rate is being compensated for by a change in the fine structure of the material. Our data can not allow us to distinguish between the two, however, either way it suggests that the cells are capable of compensating for changes in response to shear stress so as to maintain a desired level of proteoglycan activity. We have only investigated this with regard to platelet activity and further studies would be needed to determine if this compensatory mechanism, potentially important for vascular homeostasis, is general with regard to the myriad of other proteoglycan activities, as well as helpful discussions with Mathew A. Nugent (Boston University School of Medicine).

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